Wavelength-Dependent Induction of CYP24A1-mRNA after UVB-Triggered Calcitriol Synthesis in Cultured Human Keratinocytes

Michael Bär[1], Dörte Domaschke[1], Axel Meye[2], Bodo Lehmann[1] and Michael Meurer[1]

Earlier investigations in our laboratory have demonstrated that UVB irradiation of cultured human keratinocytes induces the conversion of 7-dehydrocholesterol (7-DHC) to hormonally active 1α,25-dihydroxyvitamin D_3 (calcitriol). In the research presented here, we have investigated the influence of UVB-triggered calcitriol production on gene expression of the vitamin D_3 hydroxylating enzymes catabolic 25-hydroxyvitamin-D_3-24-hydroxylase (CYP24A1), active vitamin-D_3-25-hydroxylase (CYP27A1), and 25-hydroxyvitamin-D_3-1α-hydroxylase (CYP27B1) using real-time PCR. Our results demonstrate a marked and wavelength-dependent induction of CYP24A1-mRNA in cultured human keratinocytes supplemented with 7-DHC, which parallels the spectral optimum at about 300 nm of calcitriol production as detected by HPLC and radioimmunoassay. Owing to the high sensitivity of real-time PCR, we provide evidence of a wavelength-dependent induction of CYP24A1-mRNA even in 7-DHC-deficient keratinocytes. Interestingly, we have found a strong but transient induction of CYP24A1-mRNA in non-irradiated keratinocytes, followed by accelerated cell proliferation. In contrast, UVB and calcitriol had no effect on gene expression of CYP27A1 and CYP27B1. We conclude from these experiments a constitutive gene expression of the vitamin D_3 hydroxylases, whereas the catabolic enzyme CYP24A1 is markedly regulated by UVB, calcitriol, and perhaps cell proliferation. If confirmed at protein level, these findings could have an impact on epidermal vitamin D_3 metabolism and its modulation by UVB in health and disease.

Journal of Investigative Dermatology (2007) **127,** 206–213. doi:10.1038/sj.jid.5700493; published online 10 August 2006

INTRODUCTION

Keratinocytes are the predominant cell species in the epidermis. They play a central and unique role in cutaneous vitamin D_3 metabolism. UVB irradiation of cultured human keratinocytes supplemented with 7-dehydrocholesterol (7-DHC) induces the synthesis of substantial amounts of hormonally active 1α,25-dihydroxyvitamin D_3 (calcitriol; spectral optimum at about 300 nm). Thus, keratinocytes are the only cell type known so far in which the complete pathway from 7-DHC to hormonally active calcitriol can occur (Lehmann, 1997; Lehmann *et al.*, 1998, 2000, 2001). This pathway was confirmed in human skin by microdialysis and implicates the presence of both functionally active vitamin-D_3-25-hydroxylase (CYP27A1) and 25-hydroxy vitamin-D_3-1α-hydroxylase (CYP27B1) (Lehmann *et al.*, 2003).

[1]*Department of Dermatology, Carl Gustav Carus Medical School, Dresden University of Technology, Dresden, Germany and* [2]*Department of Urology, Carl Gustav Carus Medical School, Dresden University of Technology, Dresden, Germany*

Correspondence: Dr Michael Bär, Department of Dermatology, Carl Gustav Carus Medical School, Dresden University of Technology, Fetscherstraße 74, Dresden 01307, Germany. E-mail: michael.baer@uniklinikum-dresden.de

Abbreviations: CYP24A1, catabolic 25-hydroxyvitamin-D_3-24-hydroxylase; 7-DHC, 7-dehydrocholesterol; RIA, radioimmunoassay

Received 27 February 2006; revised 19 April 2006; accepted 23 May 2006; published online 10 August 2006

It is well established that anabolic vitamin D_3 hydroxylases are constitutively expressed in cultured human keratinocytes, whereas in these cells the catabolic 25-hydroxyvitamin-D_3-24-hydroxylase (CYP24A1) shows only poor expression at the mRNA and protein level (Bikle *et al.*, 1986; Chen *et al.*, 1994; Kerry *et al.*, 1996; Ohyama *et al.*, 1996; Schuessler *et al.*, 2001; Prosser and Jones, 2004). However, enzyme activity of CYP24A1 closely follows the corresponding mRNA levels induced by the addition of exogenous vitamin D_3 or calcitriol (Schuessler *et al.*, 2001). CYP24A1 is strongly induced by calcitriol, which results in the multi-step degradation of calcitriol to less biologically active metabolites (Tanaka *et al.*, 1977; Bikle *et al.*, 1986; Chen and DeLuca, 1995; Beckman *et al.*, 1996; Ohyama *et al.*, 1996). As a highly sensitive target gene, CYP24A1 is established as an indicator for the presence of calcitriol in keratinocytes as well as for the therapeutic efficacy of vitamin D analogs in the topical treatment of psoriatic patients (Makin *et al.*, 1989; Chen *et al.*, 1994; Chen and DeLuca, 1995; Chibout *et al.*, 2003). After binding of its ligand and heterodimerization with the retinoid-X-receptor-α, the vitamin D receptor binds to vitamin D response elements in DNA promoter regions, thus initiating target gene transactivation in vitamin D receptor-positive target cells (Umesono *et al.*, 1991; Haussler *et al.*, 1995; Strugnall and DeLuca, 1997). Corresponding gene transactivation depends on several influences, including the activity of

mitogen-activated protein kinases and downstream phosphorylation status of receptors and cofactors, thus linking the genomic and non-genomic actions of calcitriol (Darwish *et al.*, 1993; Solomon, White and Kremer, 1999; Dwivedi *et al.*, 2002). Non-genomic actions are said to be mediated by a putative membranous vitamin D receptor (Norman *et al.*, 1992). Calcitriol and its analogs as well as UVB phototherapy exert antiproliferative, prodifferentiative, and immune modulatory effects on keratinocytes that have a particular impact on the therapy of hyperproliferative skin diseases such as psoriasis vulgaris (Tsoukas, Provvedini and Manolagas, 1984; Smith, Walworth and Holick, 1986; Green *et al.*, 1988; Matsumoto *et al.*, 1991; Van de Kerkhof, 1995; Reichrath *et al.*, 1997). Moreover, the vitamin D_3 pathway may be crucial for the integrity of the epidermal barrier function (Bikle *et al.*, 2004). However, the full range of UVB and vitamin D_3 effects is not completely understood. Here, we investigated the influence of monochromatic UVB and UVB-induced calcitriol synthesis in cultured human keratinocytes on mRNA expression of the hydroxylases involved in the epidermal vitamin D_3 metabolism, using real-time PCR.

RESULTS

Wavelength-dependent induction of CYP24A1-mRNA and synthesis of hormonally active calcitriol in supplemented and non-supplemented cultured human keratinocytes after irradiation with monochromatic UVB

After supplementation with 7-DHC (25 μM), keratinocytes were irradiated with monochromatic UVB (290–310 nm) at a dose of 10 mJ/cm^2. As shown in Figure 1, CYP24A1-mRNA induction starts from 8 hours after UVB exposure. At 290 and 310 nm, the corresponding mRNA expression is limited approximately to a 20-fold increase, if compared to reference expression levels, quickly entering a plateau phase. In contrast, CYP24A1-mRNA induction after irradiation at 300 nm continuously increases up to 37-fold without reaching a plateau within 24 hours. As shown in Figure 2, the corresponding *de novo* synthesis of calcitriol is highest after irradiation at 300 nm (up to 1,415 fmol calcitriol/culture dish within 24 hours), whereas irradiation at 290 and 310 nm results in lower calcitriol levels (up to 570 fmol calcitriol/culture dish within 24 hours). When keratinocytes were irradiated at a dose of 10 mJ/cm^2 in the absence of exogenous 7-DHC, the induction of CYP24-mRNA is also highest starting from 8 hours after irradiation at 300 nm, but showing only an 8-fold increase compared to the baseline expression level, whereas irradiation at 290 nm results in a delayed and

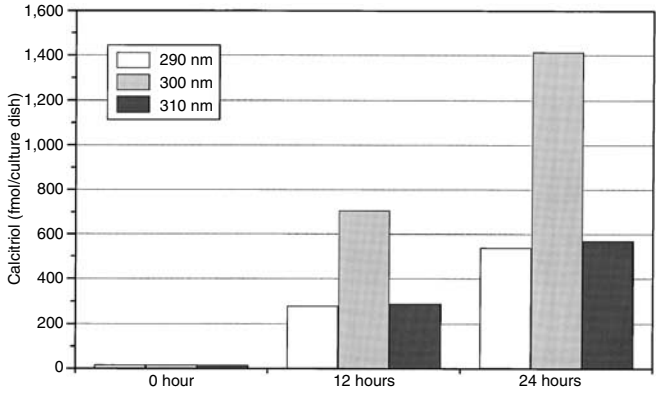

Figure 2. Wavelength-dependent production of calcitriol in keratinocytes supplemented with 7-DHC. Wavelength-dependent *de novo* synthesis of calcitriol after supplementation with 7-DHC before UVB exposure at 290–310 nm (10 mJ/cm^2) in cultured human keratinocytes. UVB irradiation with a spectral optimum at 300 nm results in maximum detectable amounts of calcitriol up to 1415 fmol/culture dish; level of calcitriol at 0 hour was below limit of detection. Data presented here are the mean of two independent experiments.

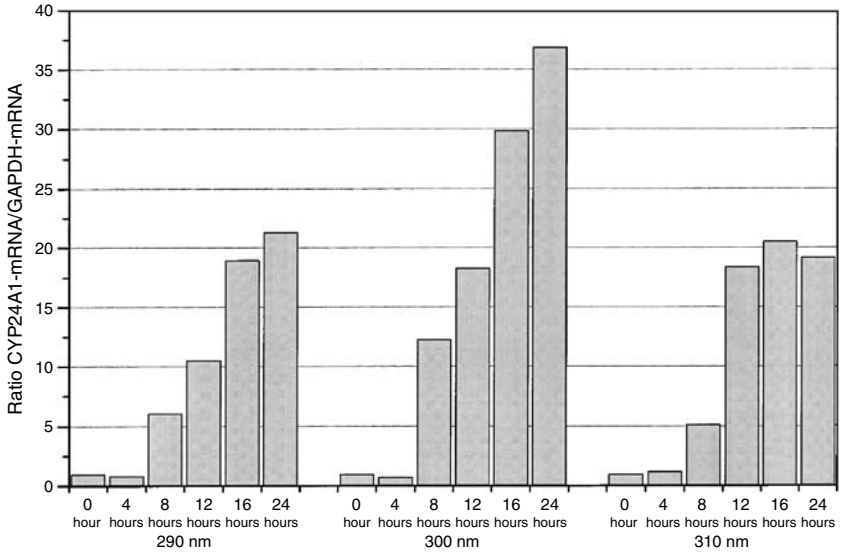

Figure 1. Wavelength-dependent induction of CYP24A1-mRNA in keratinocytes supplemented with 7-DHC. Wavelength-dependent induction of CYP24A1-mRNA starting from 8 hours after monochromatic UVB irradiation (290–310 nm, 10 mJ/cm^2, spectral optimum at about 300 nm) in cultured human keratinocytes supplemented with 7-DHC (25 μM). Standardized relative gene expression of CYP24A1 expressed as ratio of copies of CYP24A1-mRNA and copies of GAPDH-mRNA; expression level of non-irradiated control keratinocytes was defined as reference expression level = 1.0. Data presented here are based on one representative series.

low increase (up to 4-fold starting from 16 hours). After irradiation of non-supplemented keratinocytes at 310 and 320 nm, CYP24A1 expression does not differ from that of non-irradiated control keratinocytes after a normal medium change (Figures 3 and 4). The corresponding levels of calcitriol as determined by HPLC and radioimmunoassay (RIA) in these 7-DHC-deficient keratinocytes are, as expected, below the limit of detection (data not shown). An increase in the applied UVB dose from 10 to 20 mJ/cm^2 at 300 nm does not result in an additional increase in detectable CYP24A1-mRNA (8-fold), but reduces the viability of keratinocytes from about 80 to 25% (data not shown). Control experiments in which keratinocytes were incubated in the presence of exogenous calcitriol (10^{-7} M) result in a marked and prompt induction of CYP24A1-mRNA starting as soon as from 2 hours with an over 70-fold relative expression within 24 hours. Control experiments in which non-irradiated keratinocytes were supplemented with 7-DHC result in identical induction patterns as shown for non-irradiated and non-supplemented control keratinocytes (data not shown).

Constitutive mRNA expression of CYP27A1 and CYP27B1 in non-supplemented cultured human keratinocytes upon monochromatic UVB irradiation and incubation in the presence of exogenous calcitriol

Gene expression of the anabolic hydroxylases CYP27A1 and CYP27B1 is not influenced by monochromatic UVB and calcitriol, with only small and nonspecific changes around the basic mRNA expression levels (data not shown).

Transient induction of CYP24A1-mRNA in non-irradiated and non-supplemented keratinocytes associated to a normal medium change is followed by accelerated cell proliferation

As shown in Figure 4, a normal medium change with complete growth medium (Medium 154 CF/PRF + HKGS-Kit) results in a strong (up to 16-fold compared to baseline expression level) but transient increase in detectable CYP24A1-mRNA at 8 hours. This induction peak is followed by an increase in the percentage of keratinocytes going through the S phase as detected by FACS analysis (maximum 33%). In contrast, about 12% of control keratinocytes incubated without medium change pass the S phase (maximum 17%). Here, CYP24A1-mRNA expression only marginally differs from reference expression levels (Figure 4). The CYP24A1-mRNA induction associated to cell cycle changes also cannot be demonstrated after incubation in the presence of basal medium (Medium 154 CF/PRF) or basal medium that had previously been supplemented with epidermal growth factor (0.2 ng/ml) or actinomycin D (2 μg/ml, data not shown).

DISCUSSION

As shown in Figure 1, our results clearly demonstrate a marked and wavelength-dependent induction of CYP24A1-mRNA after monochromatic UVB irradiation (10 mJ/cm^2, 290–310 nm) in cultured human keratinocytes supplemented with 7-DHC. Here, CYP24A1-mRNA induction was highest at a wavelength of about 300 nm (Figure 1), thus paralleling the spectral optimum of corresponding substantial calcitriol production as detected by HPLC and RIA (Figure 2; Lehmann *et al.*, 2001). Our data are in keeping with the findings of Vantieghem *et al.* (2005), which indicate a UVB-mediated

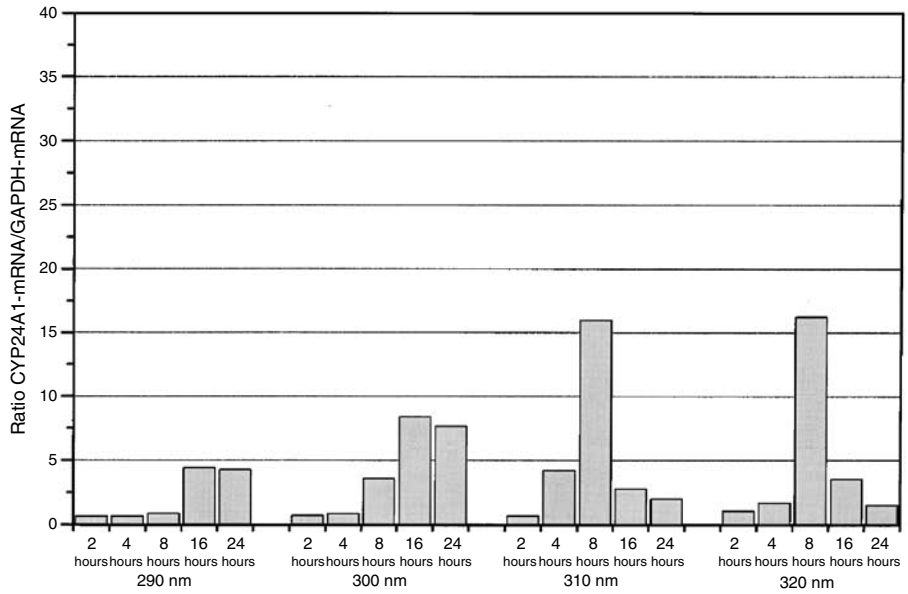

Figure 3. Wavelength-dependent induction of CYP24A1-mRNA in 7-DHC-deficient, non-supplemented keratinocytes. Wavelength-dependent induction of CYP24A1-mRNA with a spectral optimum at about 300 nm in 7-DHC-deficient cultured human keratinocytes in the absence of exogenous 7-DHC (290–320 nm, 10 mJ/cm^2). Gene expression after irradiation at 310 and 320 nm does not differ from that of non-irradiated control keratinocytes and may be based on ligand-independent induction of CYP24A1-mRNA. Standardized relative gene expression of CYP24A1 expressed as a ratio of copies of CYP24A1-mRNA and copies of GAPDH-mRNA; expression level of non-irradiated control keratinocytes was defined as reference expression level = 1.0. The data presented here are based on one representative series.

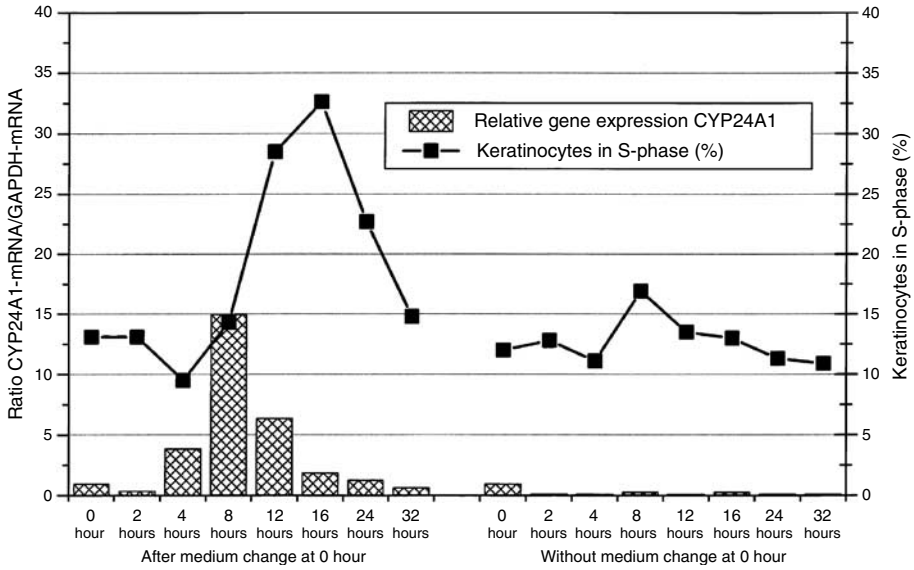

Figure 4. **Cell cycle-associated CYP24A1 gene expression after or without a normal medium change.** Cell cycle-associated transient CYP24A1-mRNA induction in non-irradiated keratinocytes after a normal medium change and constitutive CYP24A1-mRNA expression during basal proliferation activity in keratinocytes without medium change. The strong but transient CYP24A1-mRNA induction at 8 hours after a normal medium change is followed by an increased percentage of keratinocytes going through the S phase, whereas keratinocytes incubated without any medium change at 0 hour show constitutive expression levels as well as basal proliferation activity as determined by FACS cell cycle analysis: standardized relative gene expression of CYP24A1 expressed as a ratio of copies of CYP24A1-mRNA and copies of GAPDH-mRNA; expression level of non-irradiated control keratinocytes was defined as reference expression level = 1.0 (left *y* axis); percentage of keratinocytes passing the S phase (right *y* axis). Real-time PCR data presented here are based on one representative series. FACS data presented are based on one series.

CYP24A1 induction after pre-incubation in the presence of a Δ^7 reductase inhibitor starting from 8 hours after polychromatic irradiation as shown by Northern blot technique. The intracellular *de novo* synthesis of calcitriol requires substantial amounts of membranous 7-DHC. However, in contrast to keratinocytes *in vivo*, cultured keratinocytes contain only little 7-DHC. It is assumed that intracellular Δ^7 reductase is upregulated in keratinocytes in the absence of heterologous cell contact to fibroblasts, which finally accelerates the conversion of 7-DHC to cholesterol (Nemanic et al., 1983, 1985; Obi-Tabot *et al.*, 2000; Lehmann *et al.*, 2001). Thus, supplementation with 7-DHC or alternatively inhibition of its metabolism by a Δ^7 reductase inhibitor is necessary to establish *in vivo*-like conditions. CYP24A1 is well established as a highly sensitive indicator gene for the presence of calcitriol in keratinocytes (Chen *et al.*, 1994; Chen and DeLuca, 1995). This was confirmed in control experiments in which exogenous calcitriol at a pharmacological concentration of 10^{-7} M was added to cell culture, resulting in a marked and prompt CYP24A1 induction (data not shown). Owing to the high sensitivity of real-time PCR, CYP24A1-mRNA induction was even shown in 7-DHC-deficient, non-supplemented irradiated keratinocytes, indirectly demonstrating the generation of low levels of calcitriol, which are below the limit of detection of HPLC and RIA, but result in a detectable CYP24A1 upregulation starting from 8 hours after UVB exposure (Figure 3). Here, CYP24A1 expression was not changed by the application of a UVB dose of 20 mJ/cm^2 at 300 nm, but the corresponding viability of the keratinocytes was significantly altered. It has previously been shown that gene expression of involucrin in cultured keratinocytes is sensitively upregulated by calcitriol at a concentration of 10^{-12} M (Su *et al.*, 1994). Thus, cell differentiation is said to be affected even by such a low concentration of calcitriol (Regnier and Darmon, 1991; Su *et al.*, 1994; Griner *et al.*, 1999). Furthermore, our results indicate that UVB (10 mJ/cm^2, 290–320 nm) and calcitriol had no effect on gene expression of the anabolic vitamin D_3 hydroxylating enzymes CYP27A1 and CYP27B1, thus confirming earlier findings of Schuessler *et al.* (2001). In contrast to the renal 1α-hydroxylase (CYP27B1), which is tightly regulated by feedback mechanisms, the extrarenal 1α-hydroxylase is only constitutively expressed. As a consequence, there should be no relevant feedback mechanism between the systemic and epidermal vitamin D pathway that finally depends on UVB radiation. Thus, the local UVB-triggered production of calcitriol may primarily regulate epidermal cellular functions in an auto- and paracrine manner, but should not be crucial for systemic vitamin D effects. This fact is supported by recent findings of Vanhooke *et al.* (2006), which indicate that a systemic vitamin D deficiency does not stimulate epidermal synthesis of calcitriol. Surprisingly, we could detect a strong but transient CYP24A1-mRNA induction peak even in non-irradiated and non-supplemented control keratinocytes after a regular medium change. This peak is followed by an increase in the population of cells going through the S phase, which is a marker of cell proliferation and must be independent from calcitriol, as the latter is shown to induce a cell cycle arrest by interacting in the G1/S transition (Jensen *et al.*, 2001). In contrast, baseline CYP24A1 expression levels were found when keratinocytes were incubated in basal medium as well

as after incubation without medium change, indicating a causal role of growth factors. However, epidermal growth factor at a concentration commonly used in cell culture had no influence on CYP24A1 gene expression. Furthermore, the transient CYP24A1-mRNA induction peak was suppressed by actinomycin D and is therefore owing to mRNA neosynthesis. It is conceivable that this transient induction of CYP24A1-mRNA is mediated by mitogen-activated protein kinases involved in the regulation of vitamin D receptor and retinoid-X-receptor-α functions by phosphorylation of these and other receptors and their cofactors as already shown for the estrogen receptor by Kato *et al.* (2000). As a consequence, corresponding CYP24A1 transactivation may occur in a ligand-independent manner (Dwivedi *et al.*, 2002; Nguyen *et al.*, 2004), which besides its role as an epiphenomenonon may be a clue to the involvement of CYP24A1 in alternative and vitamin D-independent pathways. This is all the more interesting as mitogen-activated protein kinases are well known to be involved in cellular proliferation, differentiation, and apoptosis (Xia *et al.*, 1995; Whitmarsh and Davis, 1996; Chang and Karin, 2001). A UVB-induced cell cycle arrest as demonstrated by Courtois *et al.* (1998) might finally explain the missing transient induction of CYP24A1-mRNA after monochromatic UVB irradiation at 290 and 300 nm, respectively.

We conclude from our data a constitutive expression of the vitamin D_3 hydroxylases, whereas gene expression of CYP24A1 is markedly influenced by UVB light, the presence of calcitriol, and cell proliferation. To our knowledge, this is the first study demonstrating the wavelength-dependent induction of CYP24A1 in cultured human keratinocytes, which is a sensitive indicator of the UVB-triggered production of calcitriol in these cells. Our findings may also explain the lack of additive effects between oral or topical calcitriol and UVB phototherapy in the treatment of psoriasis, as UVB-induced CYP24A1 might result in catabolism of exogenous calcitriol (Prystowsky *et al.*, 1996). As a consequence, a modification of UVB phototherapy (e.g. by epidermal enrichment by 7-DHC supplementation or inhibition of the Δ^7 reductase activity before UVB exposure or combination with selective CYP24A1 inhibitors and CYP24A1-resistent calcitriol analogs) may optimize the therapeutic control of hyperproliferative skin disorders (Nemanic *et al.*, 1985; Morris, 1999; Schuster *et al.*, 2001; Kahraman *et al.*, 2004). Furthermore, it can be assumed that a UVB-induced calcitriol synthesis in keratinocytes not only regulates CYP24A1 expression but also other genes involved in epidermal growth and differentiation (Lu *et al.*, 2005) in a primarily auto- and/or paracrine manner, which remains to be studied in forthcoming investigations. If confirmed at the protein and enzyme level, these findings could finally enhance the understanding of the epidermal calcitriol metabolism and its modulation by UVB irradiation in health and disease.

MATERIALS AND METHODS

Cell culture and incubation conditions

Human keratinocytes from neonatal foreskin were purchased commercially (HEKn, Cascade Biologics Inc., Portland, OR) and expanded in accordance with the manufacturer's manual. Second passage keratinocytes were seeded into culture dishes (Greiner Bio-One, ∅ 34 mm, Frickenhausen, Germany) at a density of about 3.6×10^3 viable cells/cm^2. Keratinocytes were grown to a subconfluent monolayer within 5–7 days at 0.15 mM $CaCl_2$ in 2 ml of Medium 154 CF/PRF, which had previously been supplemented by the content of the HKGS-Kit (both from Cascade Biologics). The growth medium was changed every second day. Cell culture and subsequent incubations were carried out at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

UVB irradiation of cell cultures

The cell culture was rinsed twice with phosphate-buffered saline (Dulbecco's Gibco, Invitrogen, Karlsruhe, Germany) before being monochromatically irradiated through a thin layer of 1.2 ml phosphate-buffered saline at 290–320 nm (bandwidth: $\Delta\lambda = 5$ nm, Dermolum UM, Müller Elektronik-Optik, Moosining, Germany; lamp: XBO 1000, Osram, München, Germany). The mean irradiance reaching the cell surface (about 0.17 mW/cm^2) was measured by an integrated thermopile (TS 50-1, Physikalisch-Technische Werkstätten, Jena, Germany) before the irradiation experiment. The culture dish was continuously and axially turned under the UVB beam (diameter 17 mm), which was positioned radially to the rotating dish (diameter 34 mm). The effective UVB dose was chosen at a physiologically relevant dose of 10 or 20 mJ/cm^2 (Blaudschun *et al.*, 2000). After UVB exposure, keratinocytes were incubated in 2 ml of growth medium for 2 hours up to a maximum of 24 hours under incubation conditions as described above.

Pre-incubation in the presence of 7-DHC (25 μM) before UVB exposure

7-DHC (25 μM, Sigma-Aldrich Chemie, Taufkirchen, Germany) was dissolved in ethanol (0.5 vol%) before being added to growth medium that had previously been supplemented with BSA (1 vol%, Albumin bovine Fraction V Solution 7.5%, Sigma-Aldrich Chemie). After a pre-incubation period of 1 hour, keratinocytes were exposed to UVB and incubated as described above. Cells from parallel culture were used for the analytical determination of calcitriol generated in keratinocytes after UVB irradiation. Parallel control keratinocytes were incubated in the presence of 7-DHC without subsequent UVB irradiation.

Incubation in the presence of calcitriol (10^{-7} M)

Calcitriol (10^{-7} M, Teijin, Tokio, Nippon) was dissolved in ethanol (0.1 vol%) before being added to growth medium that had previously been supplemented with BSA (0.15 vol%). The incubation of keratinocytes was performed as described above.

Incubation in the presence of actinomycin D (2 μg/ml)

Actinomycin D (2 μg/ml, Sigma-Aldrich, Steinheim, Germany) was dissolved in ethanol (0.1 vol%) before being added to the growth medium. The incubation of keratinocytes was performed as described above.

Incubation in the presence of epidermal growth factor (0.2 ng/ml)

Keratinocytes were incubated under incubation conditions as described above in basal medium (Medium 154 CF/PRF without components of HKGS-Kit, Cascade Biologics) after and without

previous supplementation with epidermal growth factor (0.2 ng/ml, Sigma-Aldrich, Steinheim, Germany).

HPLC

After UVB irradiation and incubation for 12 and 24 hours, medium and detached keratinocytes were separately extracted with methanol:chloroform = 1:1. Chloroform phases were dried, residues dissolved in 200 μl eluent, and subjected to normal-phase-HPLC (Merck/Hitachi System; column: LiChroCART 250-4, Superspher Si60, 5 μm; eluent: *n*-hexane:2-propanol:methanol = 87:10:3 (v/v/v); flow rate: 1 ml/min). Calcitriol containing fractions (1 ml/min) determined after prior chromatography of a ^{3}H-calcitriol standard (t_R = 21.1 minutes) were pooled, dried under nitrogen, and analyzed for calcitriol. Calcitriol comigrated with synthetic ^{3}H-calcitriol in both normal-phase- and RP-HPLC systems (Hibar column, 250-4, LiChrospher 100RP-18, 5 μm, Merck, Darmstadt, Germany; eluent: methanol:water = 85:15 (v/v), flow rate: 1 ml/min). The data presented here are the mean of two independent experiments.

^{125}I-RIA

Calcitriol was determined by using a ^{125}I-RIA in accordance with the manufacturer's instructions (Nichols Institute, Bad Nauheim, Germany). The sensitivity of the assay was defined to be 2.1 pg/ml. The results were converted to fmol of calcitriol per culture dish. The mean number of keratinocytes per culture dish was determined as being about 4×10^5 cells (CASY1 Cell Counter & Analyzer System, Schärfe System, Reutlingen, Germany). The data presented here are the mean of two independent experiments.

Cytotoxicity assay

After incubation procedure, 1 ml of culture supernatant was asservated and stored at −20°C until measurement. Lactate dehydrogenase activity was determined colorimetrically, applying the Cytotoxicity Detection Kit (LDH, Roche, Mannheim, Germany). Cytotoxicity and subsequently viability were calculated in accordance with the manufacturer's instruction. The viability of non-irradiated cells at the end of the incubation period (24 hours) was generally more than 80%, with the exception of UVB irradiation experiments carried out at 290 nm (10 mJ/cm^2) and 300 nm (20 mJ/cm^2). Here, the corresponding end point viability was approximately 50 or 25%.

FACS cell cycle analysis

Non-irradiated keratinocytes (after and without a normal medium change) were incubated for 2 hours up to a maximum 32 hours under incubation conditions as described above. Keratinocytes from parallel cell culture were used for CYP24A1 real-time PCR assays. The adherent keratinocytes were detached by the addition of 500 μl of trypsin/EDTA (Cascade Biologics) before trypsin activity was stopped by the addition of 500 μl defined trypsin inhibitor (Cascade Biologics). The resulting cell suspension was centrifuged twice for 10 minutes at 2,000 r.p.m. and re-suspended in 1 ml of phosphate-buffered saline before the residual cell pellet was re-suspended in 1 ml buffer solution provided with the CycleTEST PLUS DNA Reagent Kit (Becton Dickinson, San José, CA) and immediately deep-frozen at −80°C. FACS cell cycle analysis was carried out in accordance with the manufacturer's protocol. For each sample, 1×10^5 isolated and purified nuclei were analyzed on an FACS flow cytometer (Becton Dickinson) after propidium iodide staining. Areas under the curve of ''DNA content peaks'' were assigned manually to the corresponding cell cycle phase (ModFit-LT 1.0, Verity Software House, Topsham, ME). The FACS data presented here are based on one series.

Isolation of total RNA

After incubation, the supernatant was removed and the cell culture was washed twice with phosphate-buffered saline. Total RNA was extracted using the Invisorb Spin Cell RNA Mini-Kit (Invitek, Berlin, Germany) in accordance with the manufacturer's recommendations. The purity and quantity of the eluated RNA were photometrically verified (Ultrospec 3000, Pharmacia Biotech, Freiburg, Germany) before being stored at −80°C until reverse transcription.

cDNA synthesis

cDNA was synthesized applying M-MLV Reverse Transcriptase RNase H Minus Point Mutant (Promega, Mannheim, Germany) in accordance with the manufacturer's protocol and additionally using oligo(dt)$_{15}$-Primer and PCR Nucleotide Mix (both from Promega).

Real-time PCR

Real-time PCR was performed using the LightCycler instrument (Roche, Mannheim, Germany). Reactions were assembled using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche) in accordance with the manufacturer's protocol. cDNA solution (2 μl) were added to 18 μl of master mix corresponding to a final reaction volume of 20 μl. Final primer concentration was set at 0.7 μM each. Primer sequences (Mitschele *et al.*, 2004): CYP24A1 – forward, 5′-gCAgCCTAgTgCAgATTT-3′ and reverse, 5′-ATTCACCCAgAACTg TTg-3′; CYP27A1 – forward, 5′-ggCAAgTACCCAgTACgg-3′ and reverse, 5′-AgCAAATAgCTTCCAAgg-3′; and CYP27B1 – forward, 5′-TgTTTgCATTTgCTCAgA-3′ and reverse, 5′-CCgggAgAgCTCATA CAg-3′ (TIB MOLBIOL, Berlin, Germany). $MgCl_2$ concentration was adjusted at 2 mM for CYP27B1 and at 3 mM for CYP27A1 and CYP24A1 assays. Annealing temperatures were: CYP24A1 = 51°C, CYP27A1 = 52°C and CYP27B1 = 53°C. The LightCycler instrument was programmed as recommended. The amplification step was followed by melting curve analysis so as to control nonspecific amplification. Melting curve reliability and primer specificity were exemplarily verified on 1.5% agarose gel electrophoresis. All samples were measured twice on independent runs. Standard curves were based on a 1:10 serial dilution of calibrated plasmid cDNA, each of which was amplified within every run (plasmid pCR 2.1, Invitrogen, Karlsruhe, Germany, inserts were cloned by Genexpress, Berlin, Germany, accession codes: CYP24A1 L13286 [1275–1609], CYP27A1 X59812 [561–852], CYP27B1 NM_000785 [839–1065]). As reference gene, the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined by RoboGene GAPDH cDNA Quantification Module (Roboscreen, Leipzig, Germany) in accordance with the manufacturer's instructions. All quantification results were expressed as a ratio of target gene and GAPDH expression. Comparability was established by standardization referring to untreated control keratinocytes, whose relative gene expression was defined 1.0 (see legends). The data presented are the mean of two independent LightCycler runs based on one representative experimental series each.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Matthias Kotzsch (Department of Pathology, Carl Gustav Carus Medical School, Dresden University of Technology, Dresden, Germany) for performing cell cycle analysis.

REFERENCES

Beckman M, Tadikonda P, Werner E, Prahl JM, Yamada S, DeLuca HF (1996) Human 25-hydroxyvitamin D_3-24-hydroxylase, a multicatalytic enzyme. *Biochemistry* 35:8465–72

Bikle DD, Chang S, Crumrine D, Elalieh H, Man MQ, Choi EH *et al.* (2004) 25 Hydroxyvitamin D 1alpha-hydroxylase is required for optimal epidermal differentiation and permeability barrier homeostasis. *J Invest Dermatol* 122:984–92

Bikle DD, Nemanic MK, Gee E, Elias P (1986) 1,25-Dihydroxyvitamin D_3 production by human keratinocytes. *J Clin Invest* 78:557–66

Blaudschun R, Brenneisen P, Wlaschek M, Meewes C, Scharffetter-Kochanek K (2000) The first peak of the UVB irradiation-dependent biphasic induction of vascular endothelial growth factor (VEGF) is due to phosphorylation of the epidermal growth factor receptor and independent of autocrine transforming growth factor α. *FEBS Lett* 474:195–200

Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. *Nature* 410:37–40

Chen KS, DeLuca HF (1995) Cloning of the human 1α,25-dihydroxyvitamin D_3 24-hydroxylase gene and identification of two vitamin D-responsive elements. *Biochim Biophys Acta* 1263:1–9

Chen ML, Heinrich G, Ohyama YI, Okuda K, Omdahl JL, Chen TC *et al.* (1994) Expression of 25-hydroxyvitamin D_3-24-hydroxylase mRNA in cultured human keratinocytes. *Proc Soc Exp Biol Med* 207:57–61

Chibout SD, De Brugerolle de Fraissinette A, Hartmann N, Picarles V, Grenet O, Cordier A *et al.* (2003) Vitamin D_3 24-hydroxylase mRNA expression in the skin of calcipotriol-treated psoriatic patients correlates with clinical efficacy. *Arch Dermatol Res* 295:269–71

Courtois SJ, Segaert S, Degreef H, Bouillon R (1998) Ultraviolet B suppresses vitamin D receptor gene expression in keratinocytes. *Biochem Biophys Res Comm* 246:64–9

Darwish HM, Burmeister JK, Moss VE, DeLuca HF (1993) Phosphorylation is involved in transcriptional activation by the 1,25-dihydroxyvitamin D_3 receptor. *Biochim Biophys Acta* 1167:29–36

Dwivedi PP, Hii CST, Ferrantes A, Tan J, Der CJ, Omdahl JL *et al.* (2002) Role of MAP kinases in the 1,25-dihydroxyvitamin D_3-induced transactivation of the rat cytochrome *P450*C24 (CYP24) promoter. *J Biol Chem* 277:29643–53

Green C, Ferguson T, Lakshmipathi T, Johnson BE (1988) 311 nm UVB phototherapy – an effective treatment for psoriasis. *Br J Dermatol* 119:691–6

Griner RD, Quin F, Jung EM, Sue-Ling CK, Crawford KB, Mann-Blakeney RS *et al.* (1999) 1,25-Dihydroxyvitamin D3 induces phospholipase D-1 expression in primary mouse epidermal keratinocytes. *J Biol Chem* 274:4663–70

Haussler MR, Jurutka PW, Hsieh JC, Thompson PD, Selznick SH, Haussler CA *et al.* (1995) New understanding of the molecular mechanism of receptor-mediated genomic actions of the vitamin D hormone. *Bone* 17:33–8

Jensen SS, Madsen MW, Lukas J, Binderup L, Bartek J (2001) Inhibitory effects of 1alpha,25-dihydroxyvitamin D3 on the G(1)–S-phase-controlling machinery. *Mol Endocrinol* 15:1370–80

Kahraman M, Sinishtaj S, Dolan PM, Kensler TW, Peleg S, Saha U *et al.* (2004) Potent, selective and low-calcemic inhibitors of CYP24 hydroxylase: 24-sulfoximine analogues of the hormone 1α,25 dihydroxyvitamin D3. *J Med Chem* 47:6854–63

Kato S, Masuhiro Y, Watanabe M, Kobayashi Y, Takeyama K, Endoh H *et al.* (2000) Molecular mechanism of a cross-talk between oestrogen and growth factor signalling pathways. *Genes Cells* 5:593–601

Kerry DM, Dwivedi PP, Hahn CN, Morris HA, Omdahl JL, May BK (1996) Transcriptional synergism between vitamin D-responsive elements in the rat 25-hydroxyvitamin D_3 24-hydroxylase (CYP24) promoter. *J Biol Chem* 271:29715–21

Lehmann B (1997) HaCaT cell line as a model system for vitamin D_3 metabolism in human skin. *J Invest Dermatol* 108:78–82

Lehmann B, Genehr T, Knuschke P, Pietzsch J, Meurer M (2001) UVB-induced conversion of 7-dehydrocholesterol to 1α,25-dihydroxyvitamin D_3 in an *in vitro* human skin equivalent model. *J Invest Dermatol* 117:1179–85

Lehmann B, Pietzsch J, Kämpf A, Meurer M (1998) Human keratinocyte line HaCaT metabolizes 1α hydroxyvitamin D_3 and vitamin D_3 to 1α, 25-dihydroxyvitamin D_3 (calcitriol). *J Dermatol Sci* 18:118–27

Lehmann B, Rudolph T, Pietzsch J, Meurer M (2000) Conversion of vitamin D_3 to 1α,25-dihydroxyvitamin D_3 in human skin equivalents. *Exp Dermatol* 9:97–103

Lehmann B, Sauter W, Knuschke P, Dressler S, Meurer M (2003) Demonstration of UVB-induced synthesis of 1alpha,25-dihydroxyvitamin D3 (calcitriol) in human skin by microdialysis. *Arch Dermatol Res* 295:24–8

Lu J, Goldstein KM, Chen P, Huang S, Gelbert LM, Nagpal S (2005) Transcriptional profiling of keratinocytes reveals a vitamin D-regulated epidermal differentiation network. *J Invest Dermatol* 124:778–85

Makin G, Lohnes D, Byford V, Ray R, Jones G (1989) Target cell metabolism of 1,25-dihydroxyvitamin D3 to calcitroic acid. Evidence for a pathway in kidney and bone involving 24-oxidation. *Biochem J* 262:173–80

Matsumoto K, Azuma Y, Kiyoki M, Okumura H, Hashimoto K, Yoshikawa K (1991) Involvement of endogenously produced 1,25-dihydroxyvitamin D3 in the growth and differentiation of human keratinocytes. *Biochim Biophys Acta* 1092:311–8

Mitschele T, Diesel B, Friedrich M, Meineke V, Maas RM, Gärtner BC *et al.* (2004) Analysis of the vitamin D system in basal cell carcinomas (BCCs). *Lab Invest* 84:693–702

Morris JG (1999) Ineffective vitamin D synthesis in cats is reversed by an inhibitor of 7-dehydrocholesterol-delta-7 reductase. *J Nutr* 129:903–8

Nemanic MK, Whitney J, Arnaud S, Herbert S, Elias PM (1983) Vitamin D3 production by cultured human keratinocytes and fibroblasts. *Biochem Biophys Res Commun* 115:444–50

Nemanic MK, Whitney J, Elias PM (1985) *In vitro* synthesis of vitamin D3 by cultured human keratinocytes and fibroblasts: action spectrum and effect of AY-9944. *Biochim Biophys Acta* 841:267–77

Nguyen TM, Lieberherr M, Fritsch J, Guillozo H, Alvarez ML, Fitouri Z *et al.* (2004) The rapid effects of 1,25-dihydroxyvitamin D_3 require the vitamin D receptor and influence 24-hydroxylase activity. *J Biol Chem* 279:7591–7

Norman AW, Nemere I, Zhou LX, Bishop JE, Lowe KE, Maiyar AC *et al.* (1992) 1α,25-Dihydroxyvitamin D_3, a steroid hormone that produces biologic effects via both genomic and nongenomic pathways. *J Steroid Biochem Mol Biol* 41:231–40

Obi-Tabot ET, Tian XQ, Chen TC, Holick MF (2000) A human skin equivalent model that mimics the photoproduction of vitamin D_3 in human skin. *In vitro Cell Dev Biol Animal* 36:201–4

Ohyama Y, Ozono K, Uchida M, Yoshimura M, Shinki T, Suda T *et al.* (1996) Functional assessment of two vitamin D-responsive elements in the rat 25-hydroxyvitamin D_3 24-hydroxylase gene. *J Biol Chem* 271:30381–5

Prosser DE, Jones G (2004) Enzymes involved in the activation and inactivation of vitamin D. *Trends Biochem* 29:664–73

Prystowsky JH, Muzio PJ, Sevran S, Clemens TL (1996) Effect of UVB phototherapy and oral calcitriol (1,25-dihydroxyvitamin D3) on vitamin D photosynthesis in patients with psoriasis. *J Am Acad Dermatol* 35:690–5

Reichrath J, Müller SM, Kerber A, Baum HP, Bahmer FA (1997) Biologic effects of topical calcipotriol (MC 903) treatment in psoriatic skin. *J Am Acad Dermatol* 36:19–28

Regnier M, Darmon M (1991) 1,25 Dihydroxyvitamin D3 stimulates specifically the last steps of epidermal differentiation of cultured human keratinocytes. *Differentiation* 47:173–88

Schuessler M, Astecker N, Herzig G, Vorisek G, Schuster I (2001) Skin is an autonomous organ in synthesis, two-step activation and degradation of vitamin D_3: CYP27 in epidermis completes the set of essential vitamin D_3-hydroxylases. *Steroids* 66:399–408

Schuster I, Egger H, Bikle DD, Herzig G, Reddy GS, Stuetz A *et al.* (2001) Selective inhibition of vitamin D hydroxylases in human keratinocytes. *Steroids* 66:409–22

Smith EL, Walworth NC, Holick MF (1986) Effect of 1α,25-dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. *J Invest Dermatol* 86:709–14

Solomon C, White JH, Kremer R (1999) Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D_3-dependent signal transduction by phosphorylating human retinoid X receptor α. *J Clin Invest* 103: 1729–35

Strugnall SA, DeLuca HF (1997) The vitamin D receptor – structure and transcriptional activation. *Proc Soc Exp Biol Med* 215:223–8

Su MJ, Bikle DD, Mancianti ML, Pillai S (1994) 1,25-Dihydroxyvitamin D_3 potentiates the keratinocytes response to calcium. *J Biol Chem* 269:14723–9

Tanaka YL, Castillo L, DeLuca HF, Ikekawa N (1977) The 24-hydroxylation of 1,25-dihydroxyvitamin D_3. *J Biol Chem* 252:1421–4

Tsoukas CD, Provvedini DM, Manolagas SC (1984) 1,25-Dihydroxyvitamin D_3: a novel immunoregulatory hormone. *Science* 224:1438–40

Umesono K, Murakami KK, Thompson CC, Evans RM (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid and vitamin D_3 receptors. *Cell* 65:1255–66

Van de Kerkhof PCM (1995) Biological activity of vitamin D analogues in the skin, with special reference to antipsoriatic mechanism. *Br J Dermatol* 132:675–82

Vanhooke JL, Prahl JM, Kimmel-Jehan C, Mendelsohn M, Danielson EW, Healy KD *et al.* (2006) CYP27B1 null mice with LacZ reporter gene display no 25-hydroxyvitamin D3-1α-hydroxylase promoter activity in the skin. *Proc Natl Acad Sci USA* 103:75–80

Vantieghem K, Kissmeyer AM, De Haes P, Bouillon R, Segaert S (2005) UVB-induced production of 1,25-dihydroxyvitamin D3 and vitamin D activity in human keratinocytes pretreated with a sterol Δ^7-reductase inhibitor. *J Cell Biochem* 98:81–92

Whitmarsh AJ, Davis RJ (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathway. *J Mol Med* 74:589–607

Xia Z, Dickens M, Raigeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270:1326–31